Bioorganic & Medicinal Chemistry 21 (2013) 2511–2517

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Covalent inhibition of SUMO and ubiquitin-specific cysteine proteases by an in situ thiol–alkyne addition

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ARTICLE INFO

Article history:
Available online 7 March 2013

Keywords:
Posttranslational modification
Cysteine peptidase
Suicide inhibitor
Catalytic triad
Electrophile

ABSTRACT

Posttranslational modification of proteins with ubiquitin and ubiquitin-like modifiers such as SUMO can be reverted by specific proteases, also referred to as deubiquitinases and isopeptidases, most of which are cysteine-dependent. We have found that the replacement of the conserved C-terminal glycine with propargylamine converts SUMO and ubiquitin to highly efficient covalent inhibitors of their cognate cysteine proteases. Attack of the catalytic cysteine onto the terminal alkyne results in the formation of a vinyl sulfide linkage. Although this reaction is reminiscent of the inhibitory mechanism of the isosteric nitrile inhibitors it was unexpected due to the low electrophilicity of the alkyne group. We show that a precise location of the functional group in the active site of the protease is crucial for the reaction, which was not inhibited by the presence of a radical scavenger. Furthermore, a mutational study of key catalytic residues in the SUMO-protease Senp1, that is H533A and D550A of the catalytic triad and Q597A as part of the oxyanion hole, revealed that these residues are not required for the observed covalent adduct formation. We therefore propose that the reaction is an in situ thiol–alkyne addition. Due to the high chemical inertness of the alkyne moiety the respective protease inhibitors should be well-suited for cellular and therapeutic applications. In keeping with this idea, selective labeling with propargylated SUMO and Ub probes was observed in lysates of cell lines expressing the cognate proteases after transient transfection.

1. Introduction

Posttranslational modifications of proteins with ubiquitin (Ub) and ubiquitin-like (Ubl) modifiers like SUMO and Nedd8 play essential roles in many cellular processes in health and disease.[1–4] These small proteins are connected via their carboxy terminus to selected target proteins, in most cases to the amino group of a lysine side chain to give an isopeptide bond. The reverse reaction is brought about by hydrolysis through specific proteases, which are also responsible for the maturation of the initially expressed Ub and Ubl precursors. About 100 deubiquitinases (also referred to as DUBs or isopeptidases), of which the majority represents cysteine-dependent enzymes, and about half a dozen of SUMO-specific proteases have been identified so far to be encoded by the human genome.[5–9] Some of these are more specialized in cleaving the isopeptide bonds, whereas others are dedicated to peptide bond hydrolysis in the maturation step. The interplay between conjugation and deconjugation is important for the dynamics and regulation of this modification as well as for Ub and Ubl recycling prior to degradation of the target protein. It is therefore not surprising that DUBs and Ubl proteases are considered promising drug targets.[10] To specifically inhibit the cysteine-dependent proteases of this group, concepts for covalent inhibitors of other therapeutically important cysteine proteases like the cathepsins and the caspases have been exploited.[11,12] To this end, various functional groups as warheads directed against the active-site cysteine were attached to the C-terminus of ubiquitin, SUMO, Nedd8 and other Ubl proteins, ranging from aldehyde[13] to nitrile,[14] vinylsulfone,[15] alkylhalide, vinylester, vinylcyanide,[16] β-lactone, benzyloxymethylketone[17] and fluoromethylketone.[18] The Ub and Ubl proteins equipped with these electrophilic groups were also extensively used for the identification of new members of the cognate proteases by labeling experiments in cell lysates.

Previously, we[19–21] and others[22] reported the site-specific attachment of Ub and Ubl modifiers to recombinant target proteins by the copper-catalyzed alkyne–azide cycloaddition (CuAAC or click chemistry).[23,24] This chemical conjugation approach serves to circumvent limitations of the enzymatic conjugation using purified proteins with regard to the difficulty to prepare defined and homogenous conjugates. While in our approaches the azide functional group was incorporated into the target protein either via cysteine derivatization[19] or by unnatural amino acid mutagenesis,[20,21] the terminal alkyne group was introduced into the Ub or Ubl protein by aminolysis of an intein-generated protein thioester with propargylamine (Pa).[19–21] In order to resemble the length of

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0968-0896
http://dx.doi.org/10.1016/j.bmc.2013.02.039

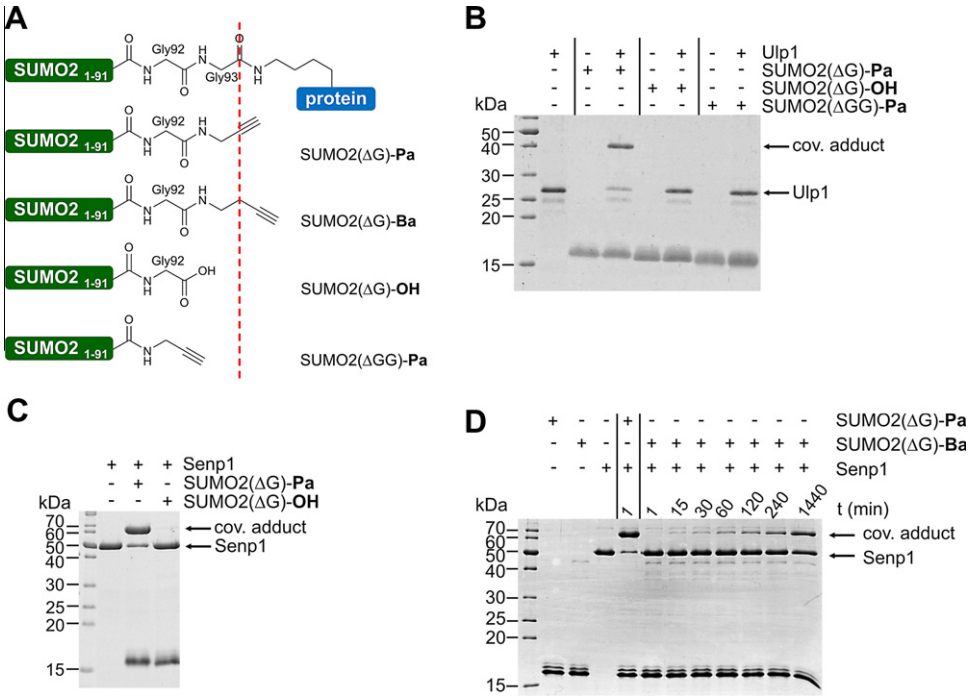

Figure 1. Propargylated SUMO2 covalently reacts with SUMO-specific proteases. (A) Schematic representation of the SUMO2 constructs used in this study in comparison to a native isopeptide-linked SUMO2. The position of the carbonyl C-atom attacked by SUMO-proteases in the isopeptide bond is marked by a red dashed line; (B) various SUMO probes were incubated with yeast Ulp1 for 1 h. The newly formed covalent adduct is indicated; (C) assays with human Senp1 carried out for 1 h; (D) time-dependent covalent adduct formation of SUMO2(ΔG)-Pa with Senp1 in comparison to SUMO2(ΔG)–Ba. All reactions in (B–D) were performed at room temperature and subsequently analyzed by denaturing and reducing SDS–PAGE (Coomassie-stained).

the resulting triazole linkage as closely as possible to that of the native isopeptide bond, we had employed Ub and SUMO constructs in which either one or both of the conserved C-terminal glycine residues were deleted (Fig. 1A). So far, alkynes have not been reported as functional moieties to inhibit cysteine proteases.[25]

Alkynes are considered bioorthogonal at physiological conditions, that is inert for reactions with biological molecules.[23,24] This is best reflected by the use of terminal alkynes for a plethora of specific cellular applications in combination with click chemistry, including the affinity-based profiling of cysteine proteases in which the alkyne group serves to attach a fluorophore or biotin moiety following the actual suicide inhibition of the enzyme.[26–29]

2. Materials and methods

2.1. General procedures and materials

For DNA cloning and protein expression standard molecular biology protocols were applied. Antibiotics were used at concentrations of 100 μg/ml (ampicillin), 50 μg/ml (kanamycin) and 34 μg/ml (chloramphenicol), respectively. Synthetic oligonucleotides were purchased from Biolegio (Nijmegen, the Netherlands). Site-directed mutagenesis was performed according to the QuikChange protocol (Stratagene). All generated plasmids were confirmed by DNA-sequencing (GATC Biotech, Konstanz, Germany and Seqlab, Göttingen, Germany). Propargylamine, 3-butynylamine and allylamine were purchased from Sigma–Aldrich, Munich, Germany. HA–SUMO2–Vinylsulfone, here termed SUMO2(ΔG)–VS, was ordered from BostonBiochem, Cambridge, USA. Monoclonal anti-HA (HA.11 Clone 16B12) and anti-EGFP antibodies were from Covance, Berkeley, USA. Polyclonal HRP-conjugated rabbit anti-mouse antibodies were used from Dako, Hamburg, Germany.

2.2. Expression plasmids

The gene fragment encoding SUMO2(ΔG)–GyrA–CBD was PCR-amplified from pSS01[20] and cloned into the *Nco*I and *Hind*III sites of pET28a (Novagen) to give pSS15 encoding HA–SUMO2(ΔG)–GyrA–CBD. A triplet codon for C-terminal Gly residue of Smt3 was introduced into pNW05 (encoding His_6–Smt3(ΔGG)–GyrA–CBD)[19] by site-directed mutagenesis to give pSS36 encoding His_6–Smt3(ΔG)–GyrA–CBD; Site-directed mutagenesis using pGST–Senp1(aa419–644) as template (this plasmid was kindly provided by Frauke Melchior (ZMBH Heidelberg, Germany)) was carried out to give pSS22, pSS23, pSS24, and pSS37 encoding the C603S, H533A, D550A, and Q597A mutants, respectively, of GST–Senp1, as well as using pUbc9[20] as a template to give pSS35 encoding the C93A mutant of Ubc9. Plasmids pNW13,[19] pSS01,[20] pSS14,[20] pNW31[20] and pNW07[19] encoding His_6–SUMO2(ΔGG)–GyrA–CBD, His_6–SUMO2(ΔG)–GyrA–CBD, His_6–SUMO2(Q98R)(ΔG)–GyrA–CBD, SBP–Ub(ΔG)–GyrA–CBD, and SBP–HA–GpD–PML_{11}, respectively, were previously published. The plasmids for bacterial expression of His_6-T7-USP5 and eukaryotic expression of HA–USP5 were kindly provided by Frauke Melchior. The plasmid encoding His_6–Ulp1 (aa403-621) was a kind gift from Kirill Alexandrov (University of Queensland, Australia). The plasmid encoding Ub(ΔG)–VMA–CBD was kindly provided by Keith Wilkinson (Emory University, Georgia, USA). The plasmid for EGFP-Senp2[30] initially created by Marry Dasso (NICHD, Bethesda, USA), was ordered from Addgene. Plasmids used for in vivo SUMOylation reactions[31] encoding Aos1/Uba2, Ubc9 and His_6–SUMO1 were kindly provided by Gerrit Praefcke (University of Cologne, Germany).

2.3. Protein expression, purification, and C-terminal modification

For the recombinant expression of proteins, *Escherichia coli* BL21(DE3) cells transformed with the respective plasmids were

used. Cells were grown in LB media supplemented with the appropriate antibiotics at 37 °C to an OD_{600} of 0.6 before protein expression was induced with 0.4 mM IPTG at 28 °C. After 4 h, cells were harvested by centrifugation, resuspended in a suitable buffer and lysed by two passages through an Avestin C5 emulsifier. Insoluble cell debris was removed by centrifugation. CBD-tagged SUMO and Ub intein constructs were immobilized on chitin beads (New England Biolabs) equilibrated with chitin column buffer (20 mM HEPES, 500 mM NaCl, 1 mM EDTA, pH 8.0). The beads were incubated with the clarified cell lysate for 1 h at 4 °C before being washed with 20 column volumes (CV) chitin column buffer. On-column thiolysis and aminolysis were initiated by the addition of chitin column buffer containing 250 mM 2-mercaptosulfonic acid and the desired amine (250 mM propargylamine, 500 mM 3-butynylamine or 500 mM allylamine). To obtain the proteins with the free carboxyl terminus 250 mM DTT was added. After incubation for 24–48 h at 4 °C under slight agitation, the cleavage efficiency was analyzed by SDS–PAGE. If uncleaved and unbound starting material was still present, the solution was passed again over fresh chitin beads. Subsequently, the solution was dialyzed against dialysis buffer (20 mM HEPES, 150 mM NaCl, pH 8.0). For the purification of GST–Senp1 constructs, the clarified cell lysate was applied twice to a GST-bind resin (Novagen, Darmstadt, Germany) equilibrated in GST-bind/wash buffer (4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.3). The column was washed with 10 CV GST-bind/wash buffer prior to elution with six times 0.5 CV GST-elution buffer (50 mM Tris, 10 mM red glutathione, pH 8.0). Fractions containing the purified protein were pooled and dialyzed against dialysis buffer supplemented with 1 mM DTT. His_6-tagged proteins were purified over a Ni-NTA-agarose column (Invitrogen) equilibrated in buffer A (50 mM Tris, 300 mM NaCl, pH 8.0) and supplemented with 10 mM imidazole. The beads were washed with 10 CV buffer A (+20 mM imidazole) and 5 CV buffer A (+40 mM imidazole). The elution was performed by the addition of six times 0.5 CV buffer A (+250 mM imidazole). Purified proteins were dialyzed against dialysis buffer. Proteins fused to an SBP-Tag were bound to streptactin sepharose (IBA GmbH, Göttingen, Germany) equilibrated with buffer W (100 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 8.0). After washing the column with 10 CV buffer W, the protein was eluted with six times 0.5 CV buffer W containing 2 mM desthiobiotin followed by dialysis against dialysis buffer. Ubc9 was purified via cation exchange chromatography according to the protocol previously reported.[32]

2.4. Covalent adduct formation

If not indicated differently, purified Ubl(ΔG) proteins C-terminally modified with propargylamine, allylamine, 3-butynylamine, or vinylsulfone were incubated with purified Ubl deconjugating enzymes or Ubc9 at room temperate in a 2:1 molar ratio at concentrations between 2 and 10 μM. Aliquots were removed from the reaction mixture at the indicated time points and the reaction was stopped by the addition of SDS sample buffer and directly boiling the samples. Pre-incubation of the proteases with 10 mM NEM was carried out for 30 min at room temperature prior to the addition of the alkyne-probe.

2.5. Mass spectrometry

The protein samples were digested with Trypsin (Promega) according to the manufacturer's protocol. The corresponding peptide mixtures were analyzed with an Agilent 1100 HPLC system coupled to a LTQ–FT–Ultra mass analyzer (Thermo Scientific) using a Phenomenex 150/3 Kinetex C18 column (2.6 μm particle size, 100 A pore diameter) at 60 °C and a flow rate of 0.2 ml/min. The gradient applied was as follows (solvent A: 0.1% TFA/water; solvent B: 0.1% TFA in MeCN): Holding 5% B for 10 min, then linear increase of the concentration of B within 150 min to 60% B followed by an increase to 90% B within 5 min and holding 90% B for additional 15 min. Exact mass spectra of the eluting peptides were generated within the FT–ICR mass analyzer at a resolution of 100,000. Additionally, data dependent fragmentation was performed within the LTQ mass analyzer. ESI-MS analysis of intact, allylamine and 3-butynylamine modified SUMO2(ΔG) was performed using the LTQ orbitrap XL (Thermo Scientific). Multiply charged ESI-MS spectra were deconvoluted to the uncharged mass.

2.6. Labeling in cell lysates

Mouse neuroblastoma N2a-cells were cultured in DMEM (Pan Biotech GmbH, Aidenbach, Germany) supplemented with 10% FCS, 1% penicillin/streptomycin, 1% L-glutamine and 1% sodium pyruvate at 37 °C under 5% CO_2. The cells were transiently transfected with the plasmids encoding HA–USP5 or EGFP–Senp2 using GeneJuice transfection reagent (Novagen) according to the manufacturer's protocol. 24 h after transfection, the cells were washed with ice cold PBS (140 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) and scraped off from the culture dish. After centrifugation, the harvested cells were incubated with 100 μl ELB buffer (50 mM Tris pH 7.5, 120 mM NaCl, 20 mM NaF, 1 mM EDTA, 6 mM EGTA, 15 mM NaPPi, 0.1% NP40) for 1 h on ice. Cellular debris was then removed by centrifugation and the supernatant was directly used for labeling experiments. The lysates were incubated with different concentrations of modified Ubl(ΔG) proteins for various periods of time at room temperature as indicated. In some cases a pre-treatment of the lysate with 10 mM *N*-ethylmaleimide (NEM) for 30 min on ice prior to the addition of the alkyne-probe was performed. The analyses of all reactions were carried out by western blotting.

3. Results and discussion

3.1. Covalent adduct formation between a propargylated SUMO and a SUMO protease

To our surprise, we observed that alkyne-functionalized human SUMO (SUMO2 isoform) with the last glycine residue deleted, termed SUMO2(ΔG)–Pa (Fig. 1A), reacted efficiently with the human SUMO protease Senp1 as well as with the yeast homolog Ulp1 to form stoichiometric adducts. The adducts were stable under the denaturing and reducing SDS–PAGE conditions and migrated at the molecular weights corresponding to the sum of both protein components (Fig. 1B and C), indicating that formation of a covalent bond had occurred. The reaction was strictly dependent on the presence of the propargyl moiety at the position of the terminal glycine, because control reactions with SUMO constructs either having the native glycine at this position and lacking the propargyl moiety or lacking both glycines failed to show similar adduct formation (Fig. 1B and C). Furthermore, a construct in which the propargyl group was replaced with an allyl moiety did not react (Fig. S1). Time-course experiments revealed that Senp1 formed the new species with SUMO2(ΔG)–Pa very rapidly with almost quantitative conversion at the first time-point investigated 30 sec after mixing both components (Fig. 1D & Fig. S2A). Ulp1 reacted somewhat slower with this heterologous SUMO isoform, but with a similar rate with its cognate SUMO probe from yeast, Smt3(ΔG)–Pa (Fig. S2B), indicating that the specific recognition between the two proteins is important for the high rate of the reaction. In order to further understand the nature of this unexpected reaction, we noticed that the position of the quaternary carbon atom of the ethynyl group in SUMO2(ΔG)–Pa corresponded to that of the carbon

atom in the peptide or isopeptide bond that is the target of the nucleophile attack by the catalytic cysteine of the protease (Fig. 1A). The effect of mislocation of the ethynyl group by just one bond length, using a butynyl instead of propargyl group in construct SUMO2(ΔG)–Ba resulted in a dramatic decrease of the rate of adduct formation (at least 480-fold; Fig. 1D; see also Fig. S3). The importance of the catalytic cysteine of the protease for this reaction was apparent from control reactions using enzymes that were either genetically inactivated by a Cys-to-Ser mutation or chemically blocked by pre-treatment with the thiol-reactive NEM. In both cases no adduct formation was observed (see Section 3.5 & Fig. 4A; Fig. S4). In contrast, pre-incubation of the protease with progargylamine in high excess (25,000-fold) did not block the reaction (data not shown). Consistent with a participation of the active-site cysteine in covalent adduct formation, SUMO2(ΔG)–Pa acted as an inhibitor of the protease Senp1 (Fig. S5).

3.2. Covalent adduct formation is also observed for ubiquitin proteases with propargylated ubiquitin

A similar adduct formation could be observed between the deubiquitinase USP5 and the corresponding Ub(ΔG)–Pa inhibitor, however, within the evaluated reaction times no cross-reactivity was detectable between the proteins taken from the SUMO and Ub pathways (Fig. S6). These results underlined the high selectivity and the generality of the reaction.

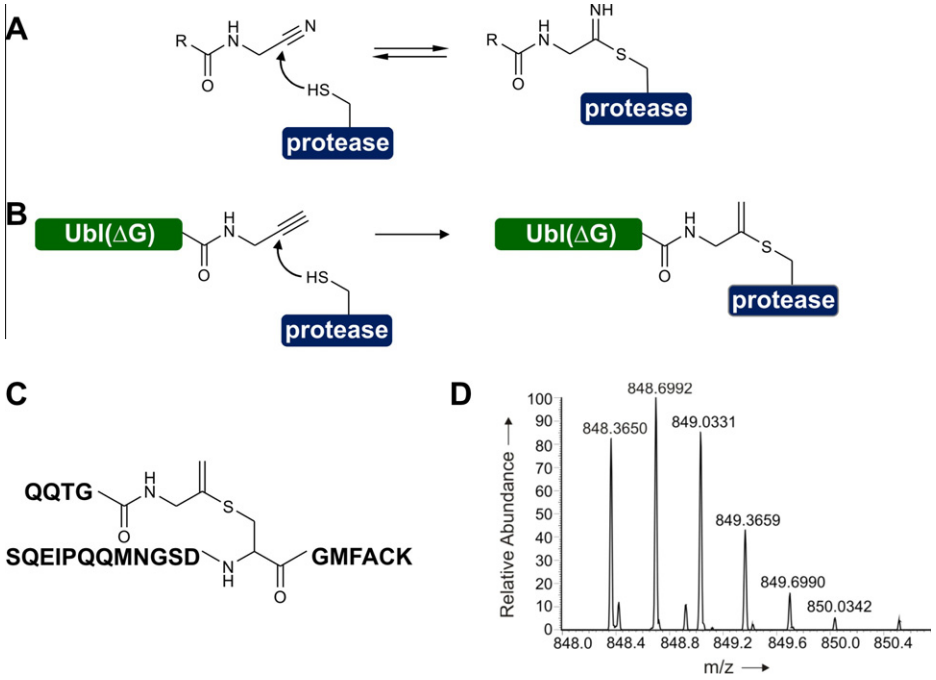

Figure 2. Mechanism of covalent inhibition by an thiol–alkyne addition. (A) The known reversible reaction of nitrile inhibitors with cysteine proteases to form a thioimidate (R = peptide or protein); (B) proposed scheme of the reaction observed here leading to a vinyl sulfide linkage; (C) structure of the fragment containing the covalent linkage obtained by a tryptic digest using the His_6–SUMO2(Q98R)(ΔG)–Senp1 adduct. The Q98R mutation was introduced into SUMO2 to produce a smaller fragment by tryspin digestion; (D) ESI-MS analysis of the fragment shown in (C). Signals for the triple charged ion: $m/z_{calc.}$ = 848.3647, $m/z_{obs.}$ = 848.3650.

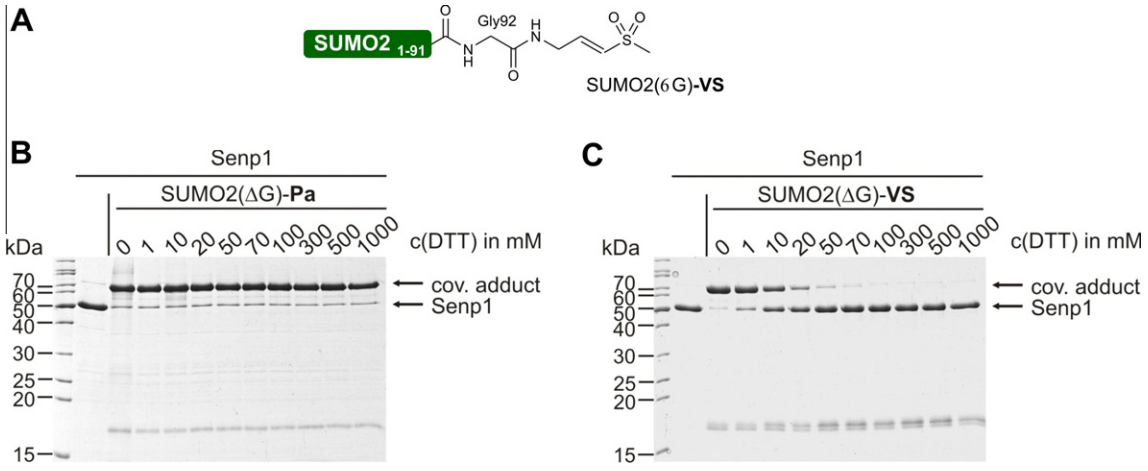

Figure 3. Comparison in reactivity of the propargylated probe with the corresponding vinylsulfone. SUMO(ΔG)–Pa does not undergo unspecific side-reactions with free thiols in contrast to SUMO2(ΔG)–VS. (A) Schematic representation of SUMO2(ΔG) C-terminally modified with a glycine–vinylsulfone (VS) moiety. SUMO2(ΔG)–Pa; (B) and SUMO2(ΔG)–VS; (C) were incubated with indicated concentrations of dithiothreitol (DTT) for 1 h at room temperature prior to addition of Senp1.

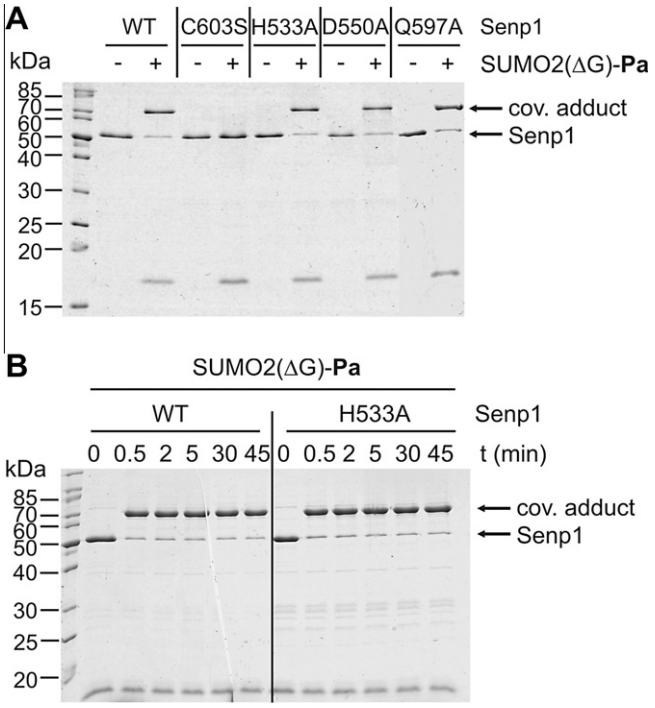

Figure 4. Impact of Senp1 active site mutations on adduct formation with SUMO2(ΔG)–Pa. (A) Protein components were incubated for 1 h at room temperature before reactions were monitored via Coomassie-stained SDS–PAGE; (B) time-dependent analysis of the reactions of SUMO2(ΔG)–Pa with wild-type (WT) Senp1 and Senp1H533A, respectively. See Figures S11 & S12 for further analyses of the mutants.

3.3. Mechanistic considerations and mass spectrometric analyses reveal formation of a vinyl sulfide linkage

Thiols are known to undergo the thiol-yne reaction with alkynes, which follows a radical mechanism.[33,34] However, the strict exclusion of light and the presence of sodium ascorbate as a radical scavenger[35] in control experiments did not impair the formation of the covalent adduct (data not shown), suggesting that this mechanism did not apply here. On the other hand, unactivated alkynes are known to undergo addition reactions with thiols in the presence of base to form the thiolate for nucleophilic attack.[36] In the case of activated alkynes with a neighboring electron-withdrawing group, the reaction can proceed at slightly basic pH with anti-Markovnikov regioselectivity and gives rise to the β-substituted vinyl sulfides.[37] The nucleophilic role of the active-site thiol side chain in cysteine proteases, which is activated by a catalytic triad (Cys–His–Asp) or at least a catalytic diad (Cys–His),[11,12] would suggest that the present reaction follows this mechanism. However, the α-substituted vinyl sulfide representing the Markovnikov product would be expected because the present alkyne moiety lacked an electron withdrawing group in the propargylated SUMO and Ub proteins and because of our observations on the importance of the positioning of the quaternary carbon atom of the alkyne group in the active site. In further support of this hypothesis, we also noticed that the chemical structure of the Ubl–Pa inhibitors and the dependence on the positioning of the alkyne moiety are reminiscent of the isosteric nitrile inhibitors. These well-studied, covalent and reversible cysteine protease inhibitors form a thioimidate[38,39] with the active-site thiolate (Fig. 2A). Taking all these considerations into account, we hypothesized that the attack of the active-site cysteine onto the terminal alkyne is a thiol–alkyne addition and results in the vinyl sulfide shown in Figure 2B. Indeed, this model was supported by mass spectrometric analysis of the covalent SUMO–Senp1 and Ub–USP5 adducts. Following the tryptic digestion of the samples, the fragments containing the new linkage could be detected and were in agreement with the calculated masses (Fig. 2C–D & Fig. S7). The identity of one fragment was further confirmed by partial peptide sequencing using MS/MS (Fig. S8). The proposed vinyl sulfide structure has also been assigned by crystal structure analysis in an independent study.[25]

We could find one report in the literature on an alkyne inhibitor tested on papain and papain-like proteases. A short dipeptide analog with an N-terminal *tert*-Butyloxycarbonyl group showed detectable inhibition of papain, yet ~2000-fold weaker than the isosteric nitrile counterpart, and was obviously not investigated any further.[40] We hypothesize that the lacking dipole moment of the alkyne group compared to the nitrile and other electrophilic groups has an impact on the binding and resident time of the inhibitor in the active site. This effect should be particularly pronounced for short peptide inhibitors with low binding affinity, but would not play a significant role for SUMO and Ub proteases.

3.4. The propargyl moiety is less reactive than commonly used electrophilic groups for protease inhibition

Surprisingly, despite the many known covalent inhibitors for cysteine proteases and their systematic variations,[11,12] an alkyne group had not been successfully reported for this purpose,[25] neither for Ub and Ubl proteases nor for cysteine proteases in general. Obviously, chemical intuition suggested that this moiety is too unreactive as an electrophile. The functional groups previously used[13–18] are typical electrophiles with graded reactivity that were selected on their ability to preferably react with an active-site cysteine and to show only little background reaction with other thiols and nucleophiles present in cellular mixtures in the relevant concentration range. To compare the reactivity of the new alkyne probes with a typical representative of this group we chose the vinylsulfone inhibitor SUMO2(ΔG)–VS (see Fig. 3A). Both probes, SUMO2(ΔG)–Pa and SUMO2(ΔG)–VS, labeled Senp1 with a similar rate during the investigated time period, with virtually complete reactions 30 sec after mixing the reactants (Fig. S9A). However, differences in reactivity became apparent when the probes were incubated with purified Ubc9 protein. This SUMO-specific E2-conjugating enzyme also employs a catalytic, yet less reactive, cysteine residue. While SUMO2(ΔG)–Pa failed to add to Ubc9 under the conditions tested, the reaction with SUMO2(ΔG)–VS showed a partial covalent adduct formation (Fig. S10). To further delineate the higher inherent disposition of the vinylsulfone probe to react with unrelated thiols, we performed a competitive quenching experiment, in which increasing amounts of dithiothreitol (DTT) were preincubated with SUMO2(ΔG)–Pa and SUMO2(ΔG)–VS before the Senp1 protease was added (Fig. 3B and C). Indeed, whereas SUMO2(ΔG)–VS was partially quenched already at 1–10 mM DTT and virtually completely inactivated at ~50 mM DTT, no effect could be observed in case of SUMO2(ΔG)–Pa for DTT at concentrations up to 1000 mM DTT (corresponding to an 100,000-fold excess over the propargyl probe).

3.5. The key catalytic residues of Senp1 for thiolate formation are not required for the thiol-alkyne addition

We next tested to what extent the key catalytic residues of Senp1 were participating in the thiol–alkyne addition. As already mentioned, the C603S mutation of the catalytic cysteine abrogated covalent adduct formation and the same result was confirmed for the SUMO2(ΔG)–VS probe (Fig. 4A & Fig. S9B), as expected. To our surprise, however, mutation of the other members of the catalytic triad, H533A and D550A,[41] had no detectable impact on

adduct formation with either probe (Fig. 4A & Fig. S9B). In particular the catalytic histidine is essential for converting the active-site cysteine side chain into the reactive thiolate. Accordingly, the H533A mutant was found to be inactive in proteolytic cleavage of a SUMOylated model protein (Fig. S11) in agreement with previous reports.[41] The inherent electrophilic nature of the vinylsulfone could explain the efficient Michael-addition even in the absence of the protease-mediated thiol deprotonation,[42] but the efficient reaction with the unactivated alkyne was unexpected. A time-course analysis of this reaction revealed that virtually quantitative adduct formation occurred already after 30 s, the first time-point taken, similar to the wild-type enzyme (Fig. 4B). The same results were obtained when the pH of the reaction mixture was lowered to pH 7 and pH 6 (Fig. S12A and B). Finally, we tested the importance of glutamine 597, which is proposed to participate in the formation of an oxyanion hole to stabilize the tetrahedral intermediate.[41,43] Also the Q597A mutant rapidly formed the covalent adduct with SUMO2(ΔG)–Pa (Fig. 4A & Fig. S12C). Together, these results suggested that these key catalytic residues of Senp1 do not provide an important contribution to the thiol-alkyne addition. When considering our observations on the strict dependence on the location of the alkyne group we conclude that the precise positioning of the two functional groups in the active site of the protease is necessary and sufficient. We therefore propose that this reaction is best described as an in situ thiol-alkyne addition, following the use of this term for other reactions that are driven by protein-mediated proximity of the two reactants.[44,45] We cannot strictly rule out potential catalytic effects from other amino acids in the active site.

3.6. Propargylated SUMO and ubiquitin can serve as specific probes for activity-based labeling in cell lysates

The high reactivity of the alkyne inhibitors and the resistance against high thiol concentrations suggested an excellent potential as suicide probes for the activity-based identification of active enzymes in complex mixtures, such as cell extracts. A mouse N2a cell line was transfected with expression plasmids for the deubiquitinase USP5 and the SUMO protease Senp2. Indeed, following addition of an HA-tagged SUMO2(ΔG)–Pa probe and an streptavidin-binding peptide (SBP)-tagged Ub(ΔG)–Pa probe to the cell lysates, selective labeling of the respective proteases could be demonstrated by western blotting (Fig. 5). Addition of NEM prior to the probe blocked the reaction (Fig. 5B). We could not detect any undesired reactions with other cellular components. Thus, the probes behaved as expected and these initial experiments further supported their usefulness for this kind of application.

4. Conclusions

We have discovered an unexpected reaction[25] between cysteine proteases of the SUMO and ubiquitin family and their propargylated substrate proteins. The formation of a vinyl sulfide linkage

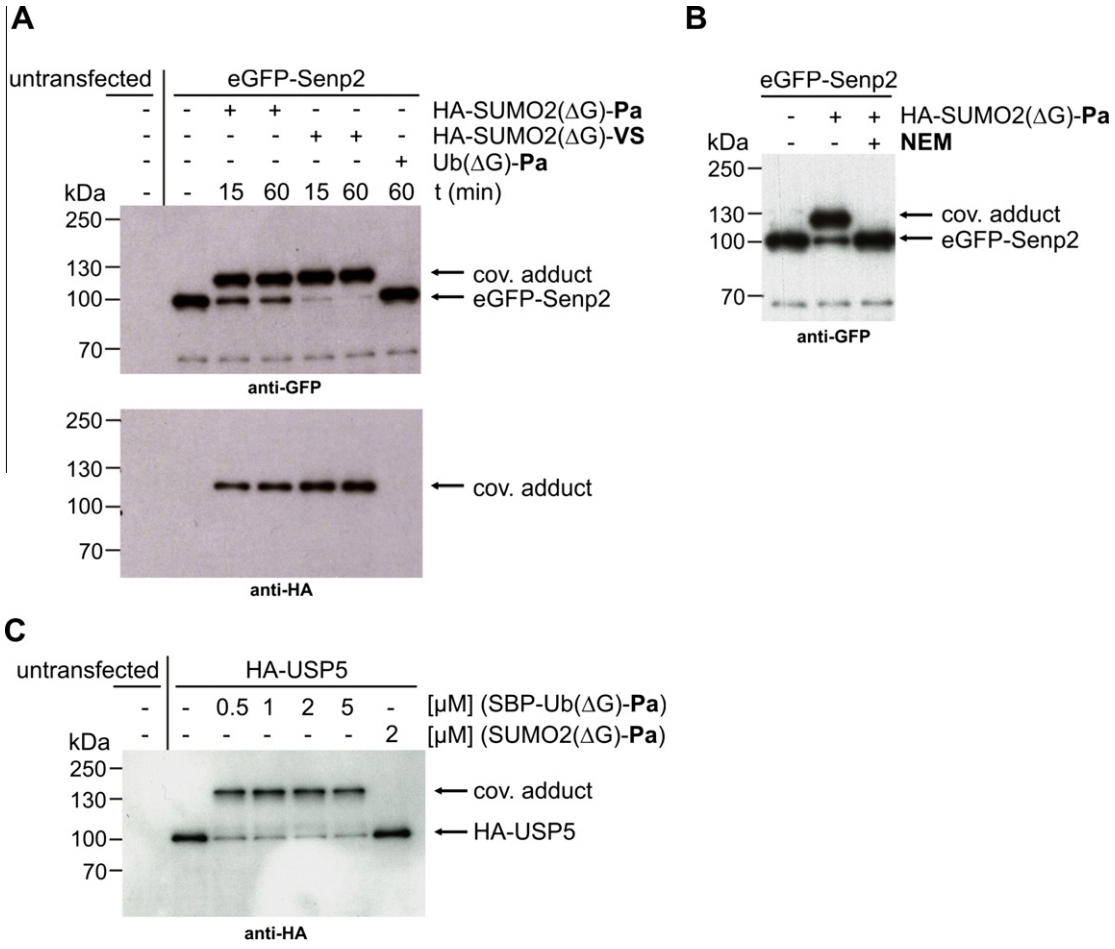

Figure 5. Specific labeling of SUMO and ubiquitin proteases in cell lysates. Mouse N2a cells were transiently transfected with plasmids expressing eGFP–Senp2 and HA–USP5. Following cell lysis, propargylated SUMO and Ub probes were added, reaction mixtures incubated at room temperature, quenched, and loaded on SDS–PAGE gels before analysis by western blotting. (A) The indicated probes were applied at of concentration of 0.5 μM. (B) the cell lysate was pre-incubated with 10 mM *N*-ethylmaleimide (NEM) prior to addition of 0.5 μM HA–SUMO2(ΔG)–Pa; (C) the probes were incubated with the cell lysate for 90 min at the indicated concentrations.

inhibited the proteases. A mutational analysis of the key catalytic residues in the active-site of the protease suggests that the thiol–alkyne addition occurs in situ and is driven by the close proximity and alignment of the two functional groups. Because of the bioinertness of the terminal alkyne group, this reaction is useful for cellular labeling reactions and is likely a valuable alternative to existing reactions that are based on stronger electrophiles. More generally, the alkyne moiety might also prove to be a powerful addition to the arsenal of medicinal chemistry building blocks for the development of cysteine protease inhibitors with therapeutic value.

Acknowledgments

We thank Lisa Kost for discussions and Patrick Helmer for technical assistance with cell transfection experiments. We are grateful to Dr. Matthias Letzel and the mass spectrometry facility for kind support with measurements. This work was funded by the Deutsche Forschungsgemeinschaft (SFB858).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.02.039.

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